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Blunted hypertrophic response in old mouse muscle is associated with a lower satellite cell density and is not alleviated by resveratrol

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Running title: Hypertrophy and satellite cell response in old and resveratrol-treated mice

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Abstract

Background: Sarcopenia contributes to the decreased quality of life in the older person. While resistance exercise is an effective measure to increase muscle mass and strength, the hypertrophic response may be blunted in old age.

Objectives: To determine 1) whether hypertrophy in the *m. plantaris* of old mice was blunted compared to adult and 2) whether this was related to a reduced satellite cell (SC) density and 3) how resveratrol affects hypertrophy in old mice.

Methods: In adult (7.5 months, n=11), old (23.5 months, n=10) and old-resveratrol-treated (n=10) male C57BL/6j mice, hypertrophy of the left *m. plantaris* was induced by denervation of its synergists. The contralateral leg served as control.

Results: After six weeks, overload-induced myofiber hypertrophy and IIB-IIA shift in myofiber type composition were less pronounced in old than adult mice (P=0.03), irrespective of resveratrol treatment. Muscles from old mice had a lower SC density than adult muscles (P=0.002). Overload-induced SC proliferation (P<0.05) resulted in an increased SC density in old, but not adult muscles (P=0.02), while a decrease occurred after resveratrol supplementation (P=0.044). Id2 and myogenin protein expression levels were higher in old than adult muscles (P<0.05). Caspase-3 was expressed more in hypertrophied than control muscles and was reduced with resveratrol (P<0.05).

Conclusion: The blunted hypertrophic response in old mice was associated with a lower SC density, but there was no evidence for a lower capacity for proliferation. Resveratrol did not rescue the hypertrophic response and even reduced, rather than increased, the number of SCs in hypertrophied muscles.

Key words: Sarcopenia; aging, muscle; hypertrophy; resveratrol; satellite cells

1. Introduction

During aging there is a progressive loss of skeletal muscle mass and function (Ballak et al., 2014; Degens and Korhonen, 2012). While resistance exercise is a widely used strategy to counteract the age-related loss in muscle mass and strength (Brown et al., 1990; Frontera et al., 1988; Leenders et al., 2013), the hypertrophic response to resistance training or overload may be blunted in old humans (Grimby et al., 1992; Kosek et al., 2006; Martel et al., 2006; Raue et al., 2009; Slivka et al., 2008) and rodents (Alway et al., 2002b; Blough and Linderman, 2000; Degens and Alway, 2003). It has been postulated that the age-related blunted hypertrophy is largely due to a decrease in the number of functional myofibers (Degens, 2012), but a reduced ability of old myofibers to hypertrophy may also contribute. The latter may be a consequence of a reduced mechanosensitivity of myofibers, a reduced number and/or ability to proliferate and/or differentiate of muscle satellite cells (SCs).

Since the activation of the AKT/mTOR pathway during overload was not diminished in muscles from old rodents, it is unlikely that a blunted rate of protein translation is a major cause of the attenuated hypertrophic response in old age (Mayhew et al., 2009). Since mechanical growth factor (MGF) and IL-4 are involved in SC activation (Aline and Sotiropoulos, 2012; Della Gatta et al., 2014), an attenuated loading-induced induction of MGF (Owino et al., 2001) and serum response factor, that controls the expression of IL-4, in old age (Lahoute et al., 2008) may result in impaired activation of SCs. This in turn could attenuate the accretion of myonuclei within the myofiber in aged muscle. SCs are thought to play an important role in the development of hypertrophy, where proliferation and differentiation of SCs prevent an excessive increase in the size of the myonuclear domain, the amount of cytoplasm controlled by a myonucleus (Van der Meer et al., 2011a). The potential importance of the addition of new myonuclei to the hypertrophying myofiber is reflected by the observation that individuals that developed the largest degree of hypertrophy in response

to a training program also had the largest accretion of new myonuclei (Petrella et al., 2006) and the highest number of SCs per myofiber before training (Petrella et al., 2008). This suggests that not only an age-related decline in SC number (Chakkalakal et al., 2012), but also impaired proliferation and/or differentiation may underlie the blunted hypertrophic response in old age. That this may indeed occur is reflected by the blunted increase in myogenin and MyoD protein expression in overloaded *m. plantaris* in old rat (Alway et al., 2002b), indicative for an impaired SC proliferation and differentiation, respectively.

The blunted hypertrophic response in old rats was associated with an increased expression of inhibitors of differentiation (Id) proteins and apoptosis (Alway et al., 2002b). An elevated expression of Id proteins is significant, as overexpression of these proteins leads to myofiber atrophy (Gundersen and Merlie, 1994), possibly by stimulating proliferation and apoptosis (Florio et al., 1998) and inhibition of differentiation of SCs via their inhibitory action on the myogenic regulatory factors, such as MyoD and myogenin (Alway et al., 2003). Furthermore, increased oxidative stress and low-grade inflammation, often observed in aged muscle (Degens, 2010), has been linked to increased expression of Id proteins (Mueller et al., 2002). In addition, TNFα can impair the transcription of MyoD and myogenin (Guttridge et al., 2000) and stimulate their breakdown in the proteasome (Langen et al., 2004) particularly when they are dimerized with Id proteins (Abu Hatoum et al., 1998). This then would hamper the transcription of muscle specific genes, as in fact observed in old rat plantaris muscle (Alway et al., 2002c).

Treatment with an anti-inflammatory anti-oxidant may restore the SC proliferation and differentiation and thereby the hypertrophic response in old age. Resveratrol (3,5,4'–trihydroxystilbene) is a polyphenol with anti-oxidant properties that has been associated with a number of health benefits. Its effect on myoblasts in particular seems to be anti-proliferative and pro-differentiation (Kaminski et al., 2012; Montesano et al., 2013; Saini et al., 2012).

This suggests that resveratrol is a potent supplement to counteract sarcopenia (Marzetti et al., 2011), enhance SC differentiation and restore the hypertrophic response to mechanical overload in old age.

Therefore, the aim of this study was threefold: 1) to test whether the hypertrophic response to a 6-week period of overload by synergist denervation was blunted in old mice compared to that in adult mice; 2) to establish whether the blunted hypertrophy was related to a reduced number of SCs and 3) to assess whether resveratrol supplementation increases the number of SCs and restores the hypertrophic response in old muscles.

2. Methods

2.1 Animals

For this study we used the following groups of male C57BL/6J mice (Janvier, France): adult (9 months old; n = 11), old (25 months old; n = 10) and old-resveratrol-treated (old-res; 25 months old; n = 10). In the left leg of the mice, the *m. plantaris* was overloaded to induce hypertrophy, while the right *m. plantaris* served as an internal control. Mice were housed individually under specific-pathogen-free conditions at 20-22 ^{0}C and a 12-h light/dark cycle. Animals were given free access to water and chow (Ssniff® S8189-S095, the same as provided at the supplier).

The mice in the old-res group received 0.4% resveratrol (98.57% pure, polygonum cuspidatum extract; 21st Century Alternative, UK) in the chow. The food intake for these mice is about 3 g per day and given a body mass of ~35 g, the daily intake of resveratrol was approximately 0.4 mg per gram body mass per day. Since the body mass did not change throughout the last six weeks prior to the terminal experiment and no difference was observed

between the old and old-res group (P=0.70), it is likely that the food intake was similar between the old and old-res group. At the age of 7.5 or 23.5 months, the *m. gastrocnemius* and *m. soleus* of the left leg were denervated to impose an overload onto the *m. plantaris* for six weeks as described previously (Degens and Alway, 2003), while the non-operated right leg served as internal control.

All experiments were approved by the local animal use and care committee of the VU University Amsterdam and conformed to the Dutch Research Council's guide for care and use of laboratory animals.

2.2 Preparation for in situ muscle function

Six weeks after induction of overload, the terminal experiment was conducted. Fifteen minutes prior to surgery, mice received a subcutaneous injection of 0.06 mL 1% Temgesic (Reckitt Benckiser, UK) as an analgesic and were anaesthetized with 4% isoflurane, 0.1 $L \cdot min^{-1}$ O_2 and 0.2 $L \cdot min^{-1}$ air. After nociceptive responses had ceased, the level of anesthesia was maintained with 1.5-2.5% isoflurane. A humidifier moistened the inhaled air to prevent dehydration due to respiration. The mice were placed on a heated pad to maintain body temperature at ~36.5 °C.

In situ force measurements were performed as described previously (Degens and Alway, 2003). The *m. plantaris* was dissected free from surrounding tissue while maintaining its innervation and blood supply. The sciatic nerve was severed and the proximal end was placed over an electrode to stimulate the muscle. The distal tendon of the *m. plantaris* was dissected free and tightened with a Kevlar thread via a small steel link to a force transducer, which was mounted on the lever arm of an isovelocity measuring system (de Haan et al.,

1989). The femur was fixed by a clamp on the condyle of the femur. During the experiment, the muscle and its surrounding were kept at 34-36 ^{0}C with a water-saturated airflow.

2.3 Experimental setup and force measurements

The order of the experiments was randomized. Contractions were elicited by supramaximal electrical stimulation of the sciatic nerve at a constant current (2 mA; 200 µs pulse width). Optimal length (ℓ_o) was defined as the muscle length where maximal tetanic isometric force was generated. To set ℓ_o, first the length at which the muscle produced maximal twitch force was determined. To fine adjust ℓ_o, tetani (150 Hz, 150 ms) were applied once every 2 min. To deplete glycogen in the fast type IIB myofibers, the muscle was subsequently stimulated for 5 min with 150-ms trains of 150 Hz once every second. Type IIB myofibers that were not glycogen depleted after this protocol were considered to be denervated myofibers. Force and length signals were digitized (1–10 kHz) and stored on disk. At the end of the experiment, the *m. plantaris* was excised, blotted dry and weighed. Then the mice were killed by cervical dislocation.

2.4 Cryosectioning

The *m. plantaris* was embedded at ℓ_o in gelatin-tyrode (NaCl, 128.3 mM; KCl, 4.7 mM; MCl_2*6H_2O, 1.05 mM; $NaH_2PO_4*H_2O$, 0.42 mM; $NaHCO_3$, 20.2 mM; EGTA, 15.0 mM; Gelatine 15% (w/v), pH 7.2) solution and frozen in liquid nitrogen. Subsequently, the *m. soleus* and *m. gastrocnemius medialis* were excised from the hind limbs and weighed.

Within a month after the contraction protocol, serial cross-sections (10 µm) were cut from the mid-belly of *m. plantaris* in a cryostat at -20 ºC. Sections were mounted on glass slides (Menzel-Gläser, superfrost® plus, GER), air-dried and stored at -80 ºC until further use. All chemicals were obtained from Sigma Aldrich (The Netherlands) unless stated otherwise.

2.4.1 Myofiber type composition

Serial sections of the *m. plantaris* were immunohistochemically stained against type I, IIA, IIX and IIB myosin heavy chain to distinguish myofiber types. Thereto, monoclonal antibodies BAD5, SC-71, 6H1 and BF-F3 (Developmental Studies hybridoma Bank, USA) against type I, IIA, IIX, IIB, respectively were used. In short, sections were fixed with acetone for 10 min at 4 ºC and washed three times for 5 min in phosphate-buffered saline (PBS) plus tween (PBST). After blocking with 10% normal swine serum for 30 min, sections were incubated with primary antibody. Subsequently, sections were washed three times in PBST for 3 min and incubated in the dark with secondary antibody (Alexa 488 anti-mouse, Molecular Probes) for 30 min. After washing with PBST, incubating with wheat germ agglutinin (WGA) (Life Technologies) for 20 min, washing with PBST and subsequently washing once more with PBS all in the dark, sections were enclosed with Vectashield®-hardset mounting medium with DAPI (Vector Laboratories, USA). The public domain software ImageJ 1.45s (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) was used to determine the number of myofibers in a cross-section, the myofiber type proportions and myofiber cross-sectional area. Myofiber outlines were manually traced.

2.4.2 Determination of denervated myofibers by PAS-staining

The glycogen content of individual myofibers was determined in 10-µm thick sections using the periodic acid-Schiff (PAS) staining (van der Laarse et al., 1992). Sections were air-dried and fixed for 5 min in 4% formaldehyde in 20 mL 0.2 M imidazole and 18 mL acetone. Subsequently, sections were oxidized in 44 mM periodic acid solution for 30 min at room temperature. After incubation, sections were briefly washed in 0.1 M HCl and stained with Schiff's reagens for 25 min at room temperature.

2.4.3 Intramuscular connective tissue content

Intramuscular connective tissue was determined using Sirius Red. Briefly, sections were air-dried and subsequently fixed for 30 min in Bouin fixative. Next, the sections were washed for 10 min with water and then incubated for 30 min in Sirius red solution saturated with picric acid. The sections were dehydrated rapidly in absolute ethanol. For a better image quality, the sections were finally cleared with xylene and mounted with Entellan. A Matlab-script (version R2012a) was used to quantify % connective tissue per picture. The RBG threshold was set at R>140, B<110, G<110, to create a binary image allowing to filter all red pixels. The same threshold was applied to all images.

2.4.4 Quantification of myonuclei and SC number

Sections were co-stained with DAPI and anti-Pax7 antibody according to (van der Meer et al., 2011b). Briefly, sections were fixed in 4% formaldehyde PBS for 10 min, washed with PBST and blocked in 10% normal donkey serum (NDS) in PBS for 30 min. Subsequently, sections were incubated in a 0.1% bovine serum albumin (BSA)-PBS solution

containing 4 µg·mL^{-1} Pax7 antibody (Developmental Studies hybridoma Bank, USA) in the dark for 60 min, washed in PBST, incubated with Alexa Fluor 488 (1:500, Molecular Probes, Life Technologies) donkey anti-mouse secondary antibody (Invitrogen, Breda, The Netherlands) and washed with PBST. Then the sections were incubated in the dark for 20 min with Texas red-WGA (1:50) conjugate (Invitrogen, Breda, The Netherlands) and washed with PBS. Finally, the sections were enclosed with Vectashield®-hardset mounting medium with DAPI (Vector Laboratories, USA). The images were captured at 10x magnification using a CCD camera (PCOl Sensicam, Kelheim, Germany) connected to a fluorescence microscope (Axiovert 200M; Zeiss, Göttingen, Germany) with image processing software Slidebook 4.1 (Intelligent Image Innovations, Denver, Colorado). The number of myonuclear fragments per myofiber cross-section were counted manually in an average of ~70 myofibers per muscle, while SC fragments per myofiber cross-section were counted throughout the whole muscle.

2.4.5 Western blots

Frozen muscle tissue (~5-10 mg) was homogenized with a Polytron mixer in ice-cold buffer (1:40, w/v) [50 mM Tris-HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 0.1 % Triton-X 100 and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and immediately stored at -80°C. The protein concentration was measured using the DC protein assay kid (Bio-Rad laboratories, Nazareth, Belgium). Forty µg of proteins were separated by SDS-PAGE (10-12% gels) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% non-fat milk for 1 h and afterwards incubated overnight (4°C) with the following

antibodies (1:1000): MyoD, Myogenin, Id2 (BD Biosciences, Oxford, UK), caspase-3, caspase-9 and eEF2 (Cell Signaling, Leiden, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (1:10,000) or anti-rabbit (1:5,000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap software (Syngene, Cambridge, UK), respectively. The results are presented as the ratio protein of interest/eEF2. eEF2 was used as a loading control as preliminary experiments showed that eEF2 expression was stable across the different treatments and conditions.

2.5 Statistics

To test for differences between age and treatment groups we used a univariate ANOVA with factors age and overload, or resveratrol and overload. Effects were considered significant at $P<0.05$. Data are expressed as means ± standard error of the mean (SEM). All calculations were performed using IBM SPSS version 20.

3. Results

3.1 Mice and muscle characteristics

Body mass, muscle length, myofiber number, number of denervated myofibers and connective tissue were not significantly affected by age, overload or resveratrol treatment (Table 1). In all groups, masses of the denervated *gastrocnemius medialis* and *soleus* muscles were significantly smaller than those of the contralateral leg ($P<0.001$). *M. plantaris* mass was lower in aged mice compared to that in adult mice ($P<0.01$). The overloaded *m. plantaris* were ~30% heavier than control muscles ($P<0.001$), irrespective of age or resveratrol

treatment. In addition, overload induced an increase in muscle cross-sectional area (P<0.001; Table 1).

3.2 Muscle function

Maximal isometric muscle force was 13% lower in muscles from old than adult mice (P=0.02) and was 27% higher in overloaded than in control muscles (P<0.001; Fig. 1A). The absence of an interaction between age and overload indicates that the increase in force was similar in adult and old overloaded muscles. Resveratrol had no effect on maximal isometric muscle force. Specific tension was lower in the *m. plantaris* from old than adult mice (P<0.01). The significant interaction between age and overload is reflected by a lower specific tension in overloaded adult muscle (P=0.047), but not old muscles.

In both adult and old muscle, the pooled myofiber CSA (FCSA) increased during overload (P=0.01), while resveratrol had no significant effect. The increase in pooled FCSA was, however, more in adult than in old *m. plantaris* (Age*Overload interaction; P=0.03; Fig. 2A). This Age*Overload interaction was also shown for each individual myofiber type (Age*Overload P<0.05; Figs. 2B-D). In addition, in all groups the hypertrophic response was larger for IIA myofibers (56%), than IIX (40%) and IIB (21%) myofibers. Note that the proportion of type I myofibers was so small (<1%) that it was left out of all analyzes.

The proportion of type IIB myofibers decreased with overload, but less so in old than adult mice (Fig. 3; Age*Overload P=0.013). This decrease in the proportion of type IIB myofibers was accompanied by an increase in the proportion of hybrid (type IIA/X and IIX/B) (Table 1; P<0.001) and in adult also type IIA myofibers (Fig. 3; Age*Overload P=0.002). Resveratrol had no significant impact on myofiber type proportions (Fig. 3).

Examples of whole muscle myofiber type staining for adult (Figs. 3D-F), old (Figs. 3G-I) and old-res (Figs. 3J-L) are provided.

3.3 Effects of age and resveratrol on SC number

The number of SCs per muscle CSA (mm^2) or SC density, was higher in adult than old muscle (Fig. 4E; P=0.002). The number of SCs per myofiber tended to be higher for adult compared to old muscle (Fig 4F; P=0.06). Hypertrophy was accompanied by an increase in the number of SCs per myofiber in both adult and old mice (Fig 4F; P<0.05). However, only in old, but not adult, muscle hypertrophy was accompanied by an increase in SC density (Fig 4E: Age*Overload P=0.02). In old-res mice, the number of SCs per myofiber (Fig 4F; Resveratrol*Overload P=0.044) and SC density (Resveratrol*Overload P=0.005; Fig. 4E) had decreased, rather than increased in the hypertrophied muscles. The number of myonuclei per fiber had increased in overloaded adult and old muscles (Fig. 4G; P<0.01), but not in overloaded old-res muscles.

3.4 Proteins related to SC proliferation and differentiation

MyoD protein expression was not significantly affected by age, overload or resveratrol (Fig. 5A). Myogenin and Id2 protein expression were higher in old compared to adult muscle, but did not significantly change with hypertrophy or resveratrol treatment (P<0.05; Fig 5B & C). In all groups, overload was accompanied by an increased expression of total caspase-3 (P<0.05; Fig. 5D), while resveratrol induced a decrease in the expression of caspase-3 expression (Fig. 5D; P<0.05). No cleaved caspase-3 was detected. There were no

significant effects of age, overload or resveratrol on caspase-9 expression in the *m. plantaris* (Fig. 5E; P>0.05).

4. Discussion

The main finding of this study is that the *m. plantaris* of 25-month-old male C57BL/6j mice showed a blunted hypertrophic response to a 6-week overload stimulus. In addition, the type IIB to IIA myofiber type transition during hypertrophy was less pronounced in the *m. plantaris* of old than of adult mice. The blunted hypertrophic response was not attributable to a lower proliferative capacity of SCs in old mice, but may rather be a consequence of the lower SC density in old than in adult muscles. Resveratrol may have decreased proliferation and/or differentiation, as reflected by a lack of increase in response to overload in SC density and number of myonuclei per myofiber in old-res mice. In contrast to our hypothesis, in old muscle resveratrol did not rescue the attenuated hypertrophy to overload.

4.1 The hypertrophic response in adult and old mice

In this study overload was induced in the left *m. plantaris* of 7.5- and 23.5-month-old male mice by denervation of its synergists. This technique has previously been shown to be an effective way to induce hypertrophy in rodents, with less inflammation than in, for instance, overload induced by ablation of synergistic muscles (Lowe and Alway, 2002). Here we show that the anatomical CSA of the *m. plantaris* increased by only 20% in the old, in contrast to the 40% increase in anatomical CSA in adult mice, while fiber numbers were similar in the plantaris muscles in adult and old mice. The blunted hypertrophy in old mice

was thus not attributable to a lower number of myofibers, but rather to blunted hypertrophy of the existing myofibers, similar to what has been observed in older people (Kosek et al., 2006). Interestingly, the hypertrophic response in IIA myofibers exceeded that of IIX and IIB myofibers in both adult and old animals. This is in agreement with a report, studying overload in 4-month-old female Wistar rats (Degens et al., 1995b). In addition, overload decreased the proportion of type IIB myofibers, however more so in adult than in old muscle. Thus, not only the development of myofiber hypertrophy, but also the transition of myofiber types during hypertrophy is partly lost in 25-month-old compared to adult mice.

4.2 The effect of mechanical overload on muscle force generating capacity in adult and old mice

Resistance exercise in both adult and old humans is associated with an increase in maximal force that is proportionally larger than the increase in muscle mass (Degens et al., 2009; Erskine et al., 2011; Jones et al., 1989; Morse et al., 2005). Here we did not find such an increase in specific tension with overload in neither adult nor old mouse muscles, similar to what has been reported before in rats (Degens et al., 1995a; Roy et al., 1982). It remains to be seen what causes this discrepancy, but part of the apparent increase in specific tension in human muscles is attributable to improved maximal voluntary activation (Erskine et al., 2011; Morse et al., 2005), which does not play a role in the maximally stimulated isolated muscle preparation in mice (Degens, 2012).

4.3 SC number during overload in adult and old mice

Many studies have shown that overload-induced hypertrophy is accompanied by the acquisition of new myonuclei, requiring proliferation and differentiation of SCs (Allen et al., 1999; Van der Meer et al., 2011a). An explanation for a blunted myofiber hypertrophy in aged muscle may thus be a lower number, a decreased mechanosensitivity, impaired proliferation and/or differentiation of SCs. Here we found that the SC density was lower in old than adult muscle, which is in accordance with others (Day et al., 2010). Despite the age-related reduction in SC density, overload did induce SC proliferation not only in adult, but also in old muscles. Similarly in old human muscle SC proliferation and hypertrophy have been observed in elderly men after resistance training (Verdijk et al., 2009). In our study, the proliferation was such that the SC density remained constant in adult, while it was even increased in old hypertrophied muscles. The observation that the SC density in old hypertrophied muscles was even elevated above that of the age-matched control muscles indicates that SC proliferation was likely not the limiting factor for the development of hypertrophy in the old muscles. Rather, it suggests that the lower absolute number of SCs and/or impaired SC differentiation and SC apoptosis contributed to the impaired hypertrophic response in aged muscle.

4.4 Expression of proteins related to SC proliferation and differentiation

An elevated expression of Id proteins is a crucial step for SC proliferation (Lluís et al., 2006), but expression beyond a certain threshold, depending on cell environment, may cause apoptosis rather than proliferation (Yokota and Mori, 2002). Even though the number of SCs was lower in old than adult muscle, the expression of Id2 proteins was elevated. This has also been observed in muscles from old rats and in that case elevated Id2 expression levels were associated with apoptosis and blunted hypertrophy (Alway et al., 2002c). Here, however, we did not find evidence of increased apoptosis in old muscles, as reflected by

similar caspase-3 expression in adult and old muscles. Therefore, it is possible that the increase in myogenin served to stimulate differentiation to compensate for an impaired function of SCs in the old muscles. Interestingly, it has been observed previously that the most atrophied muscles in old rats exhibited the largest increase in MRF and IGF-I mRNAs, suggesting no problem with the regenerative drive (Edstrom and Ulfhake, 2005), which corresponds with our data.

In contrast to previous observations in overloaded rat muscles (Alway et al., 2002b), we did not observe an overload-induced increase in MyoD and myogenin in the adult mice. It should be noted, however, that in that study (Alway et al., 2002a) the muscles were overloaded only for 2 weeks, the period of muscle growth, while we studied them when they had reached a new steady state at 6 weeks after induction of overload (Degens et al., 1995b).

In contrast to previous studies in humans (Whitman et al., 2005) and rodents (Pistilli et al., 2006; Phillips & Leeuwenburgh, 2005) we did not find evidence for increased apoptosis in muscles of the old mice, as reflected by similar caspase-3 expressions in adult and old mice. Nevertheless, in both adult and old muscles caspase-3 was significantly elevated during hypertrophy. This may reflect previous and repeated activation of the pathway. In addition, overload induced a considerable IIB-IIA myofiber type transition and a concomitant increase in hybrid myofibers in the absence of a change in myofiber number. This implies that in individual myofibers type IIB myosin must be broken down to be replaced by type IIA or IIX myosin. While the ubiquitin proteasome pathway plays an important role in the breakdown of muscle proteins, it is thought that caspase-3 is involved in the breakdown of large myosin molecules (Powers et al., 2007) and first cleaves the myosin molecule in smaller fragments that can subsequently be broken down in the proteasome (Du et al., 2004). It is thus possible that the increase in caspase-3 in hypertrophied muscles is a

prerequisite for the breakdown of type IIB myosin (in this case) and hence the observed myofiber type transition during hypertrophy.

4.5 Resveratrol decreases the SC density after overload

Oxidative stress and systemic inflammation are factors that contribute to the age-related muscle wasting and weakness (Degens, 2010). Resveratrol has been shown to prevent TNF-α induced muscle atrophy (Wang et al., 2014) and to alleviate oxidative stress also in old mice (Jackson et al., 2011). Therefore, we investigated whether resveratrol attenuates the age-related muscle wasting and normalizes muscle hypertrophy in old mice. In the present study, muscle mass, force and specific force were not affected by resveratrol. Furthermore, the hypertrophic response was not rescued by 0.4% resveratrol supplementation in the old mice. This is in accordance with others, who found that 0.05% resveratrol supplementation was not sufficient to attenuate the age-related muscle wasting even though it alleviated oxidative stress (Jackson et al., 2011). It thus appears that resveratrol does not have beneficial effects on muscle mass or quality in 25-month-old mice at a dose of 0.4%.

Resveratrol may have decreased proliferation and/or differentiation, as reflected by a lack of increase in response to overload in SC density and number of myonuclei per myofiber in old-res mice. In contrast to our hypothesis, in old muscle resveratrol did not rescue the attenuated hypertrophic response to overload. Note, however, that despite the lack of increase in myonuclear number in old-res muscle, the hypertrophic response was similar to that in the untreated old muscle. The preserved hypertrophy suggests that the increase in myonuclei was not required to induce a ~20% hypertrophy by overload, which is in line with the observation that even in SC depleted muscles myofiber hypertrophy was not attenuated (McCarthy et al., 2011). Although resveratrol has been reported to stimulate myoblast

differentiation (Kaminski et al., 2012; Montesano et al., 2013) and to block the proliferation (Montesano et al., 2013) we did not find an altered expression of Id2, MyoD and myogenin, important regulators of SC proliferation and differentiation. Resveratrol did, however, decrease total caspase-3 protein content, which is in accordance with others where resveratrol decreased mitochondrial caspase-dependent apoptotic signaling pathways (Bennett et al., 2013; Marzetti et al., 2011). However, the total caspase-9 protein content (mitochondrial dependent apoptotic pathway) was not affected by resveratrol. Therefore, in 25-month-old mice, resveratrol seems to block proliferation and subsequent differentiation of SCs after overload independent of MyoD or myogenin.

5. Conclusion

In conclusion, the *m. plantaris* of 25-month-old male mice showed a blunted hypertrophic response, in terms of an increase in myofiber size and myofiber type transition compared to the *m. plantaris* from 9-month-old mice. The blunted myofiber hypertrophy could partly be explained by an age-related decrease in SC density. Resveratrol did not alleviate the age-related decrease in muscle force, specific tension or mass. Nor did it restore the ability to develop hypertrophy to levels observed in adult mice. In the contrary, resveratrol blunted the SC response to overload in 25-month-old mice, possibly caused by a diminished proliferation and differentiation of SCs.

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Disclosure statement

The authors declare no conflicts of interest, financial or otherwise. The sponsor was in no way involved in the design or execution of this study.

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Table 1. Mice' characteristics

	Adult mice (n=11)		Old mice (n=10)		Old-res mice (n=10)	
	Control	Experimental	Control	Experimental	Control	Experimental
Body mass (gr)	36.1±3.0		35.6±0.9		35.9±1.2	
Muscle mass (mg)						
M. plantaris	24.2±0.7	32.1±1.2$^{*/o}$	22.4±0.7^{a}	28.1±1.1$^{*/o}$	23.8±0.8	29.8±0.9$^{*/o}$
M. soleus	13.3±0.3	8.9±0.4$^{*/d}$	12.2 ±0.4^{a}	8.8±0.4$^{*/d}$	13.2±1.5	9.3±0.7$^{*/d}$
M. gastrocnemius	65.2±1.8	26.5±0.6$^{*/d}$	60.1 ±1.7^{a}	28.1±0.8$^{*/d}$	58.7±2.8	28.5±2.0$^{*/d}$
Muscle length (mm)	12.8±0.4	12.8±0.4	12.9±0.4	13.0±0.3	12.3±0.3	12.7±0.3
Muscle CSA (mm^2)	1.83±0.12	2.61±0.16*	2.08±0.10	2.48±0.12*	2.07±0.10	2.53±0.17*
Myofiber number (#)	1027±58	1098±67	1104±55	1299±73	1131±66	1235±47
Ratio muscle/body mass ($mg \cdot g^{-1}$)	0.67±0.04	0.89±0.03*	0.63±0.03^{a}	0.77±0.05*	0.67±0.02	0.84±0.03*
Connective tissue (%)	11.6±1.1	12.0±1.3	16.4±1.8	14.1±1.4	12.8±1.3	11.6±1.7
Hybrid myofibers (%)	11.2±2.4	27.2±4.7*	6.4±3.1	20.2±3.6*	8.7±1.9	22.8±5.2*
Denervated myofibers (%)						
%total	1.2±0.4	0.8±0.1	1.3±0.4	1.1±0.3	1.3±0.3	0.9±0.2
%IIB	2.3±0.7	3.8±1.0	3.5±1.1	2.7±0.6	2.7±0.8	2.5±0.6

Values are mean±SEM. [a] significantly different compared with adult muscle (P<0.05), [*] significantly different compared to control muscle (P<0.05). For the muscles in the experimental group the *m. plantaris* was overloaded [o], while the *m. soleus* and *m. gastrocnemius* were denervated [d].

Figure legends

Figure 1. Effects of aging and resveratrol on maximal isometric force. **(A)** Maximal isometric force (N) of rat *m. plantaris* muscle during a 150-Hz isometric tetanus and **(B)** specific tension ($N \cdot mm^{-2}$) of the *m. plantaris* of male adult (9 months old), old (25 months old) and old-res C57Bl/6 mice. **(A)** A main age effect was observed for force ($P<0.05$), while resveratrol had no effect ($P>0.05$). Force was increased by overload in all groups ($P<0.001$). **(B)** Muscle specific force was higher for adult than for old ($P<0.01$), while resveratrol had no effect on muscle specific force ($P>0.05$). An Age*Overload interaction ($P<0.05$) was observed for specific tension, which indicates that a reduction in specific tension after overload in adult but not in old *m. plantaris* had occurred. *: different from control. [a]: different from adult. Values are mean±SEM.

Figure 2. Pooled and individual myofiber CSA for adult, old and old-res *m. plantaris* of C57Bl/6 mice. **(A)** Overload increased myofiber CSA (μm^2) in all groups ($P<0.001$), however this increase was higher in adult than in old muscle (interaction effect Age*Overload $P=0.032$). **(B)** Type IIA, **(C)** X and **(D)** B myofibers hypertrophied after overload for all groups, however the magnitude of this increase was higher for adult muscle than old (interaction effect Age*Overload for all three myofibers types $P<0.05$). *: different from control. Values are mean±SEM.

Figure 3. The proportion of type IIA, IIX and IIB myofibers inside the *m. plantaris* of adult, old and old-res C57Bl/6 mice. **(A)** No effect of age or resveratrol was observed in the proportion of type IIA myofibers. **(B)** No effect of age, resveratrol or overload was observed for the proportion of type IIX myofibers. **(C)** In both adult and old-res mice the proportion of type IIB myofibers decreased after overload ($P<0.01$), but not for old. Overload increased the proportion of IIA myofibers and decreased the proportion of IIB myofibers more in adult than

old muscle (interaction effect Age*Overload P<0.01 and P<0.05, respectively). Examples of immunohistochemical staining for type IIA, IIX and IIB myofibers in adult (Figs. 3D-F), old (Figs. 3G-I) and old-res (Figs. 3J-L) muscles are provided.*: different from control. Values are mean±SEM.

Figure 4. Effects of aging, overload and resveratrol on SC density. SCs were identified in plantaris muscle cross-sections by co-localization of DAPI and staining of Pax7 of C57Bl/6 mice. Example of a section stained for **(A)** Pax7 (SC; green) and DAPI (nuclei; blue), (**B**) Pax7 (green) and WGA (membranes; red), (**C**) DAPI (blue) and WGA (red) and (**D**) Pax7 (green), DAPI (blue) and WGA (red) merged. Arrows indicate SCs. Bar represents 50 µm. **(E)** Number of SC fragments expressed per muscle CSA (mm^2) were higher for adult than for old muscle (P<0.01). A significant Age*Overload interaction (P<0.05) is reflected by an increase in the number of SC fragments per mm^2 for old, but not for adult muscle. Resveratrol decreased the number of SC fragments per mm^2 after overload (interaction effect Resveratrol*Overload P<0.01). **(F)** Number of SC fragments expressed per myofiber cross-section tended to be higher in the adult than old group (P=0.06). Overload increased the number of SC fragments per myofiber cross-section in both adult and old, but not in old-res mice (interaction effect Resveratrol*Overload P>0.05). (**G**) Overload increased the number of myonuclear fragments per myofiber cross-section in adult and old (Fig. 4G; P<0.01), but not for old-res muscles (P>0.05). *: different from control. [a]: different from adult. [b]: different from old. Values are mean ± SEM.

Figure 5. Protein content in *m. plantaris* for MyoD, myogenin, Id2 and caspase-3. **(A)** MyoD protein expression was not affected by age or overload (P>0.05). **(B)** Myogenin and **(C)** Id2 protein content were higher in old muscle than in adult muscle (P<0.01), but unaffected by resveratrol (P>0.05). **(D)** Capase-3 protein content was increased by overload in all groups (P<0.05) and decreased by resveratrol (P<0.05). (**E**) No age-, overload- or resveratrol-

induced changes were observed for caspase-9 expression. (**F**) Western blot examples for MyoD, myogenin, Id2, caspase-3, caspase-9 and eEF2 protein expression. *: different from control. [b]: different from old. Values are mean±SEM.

1

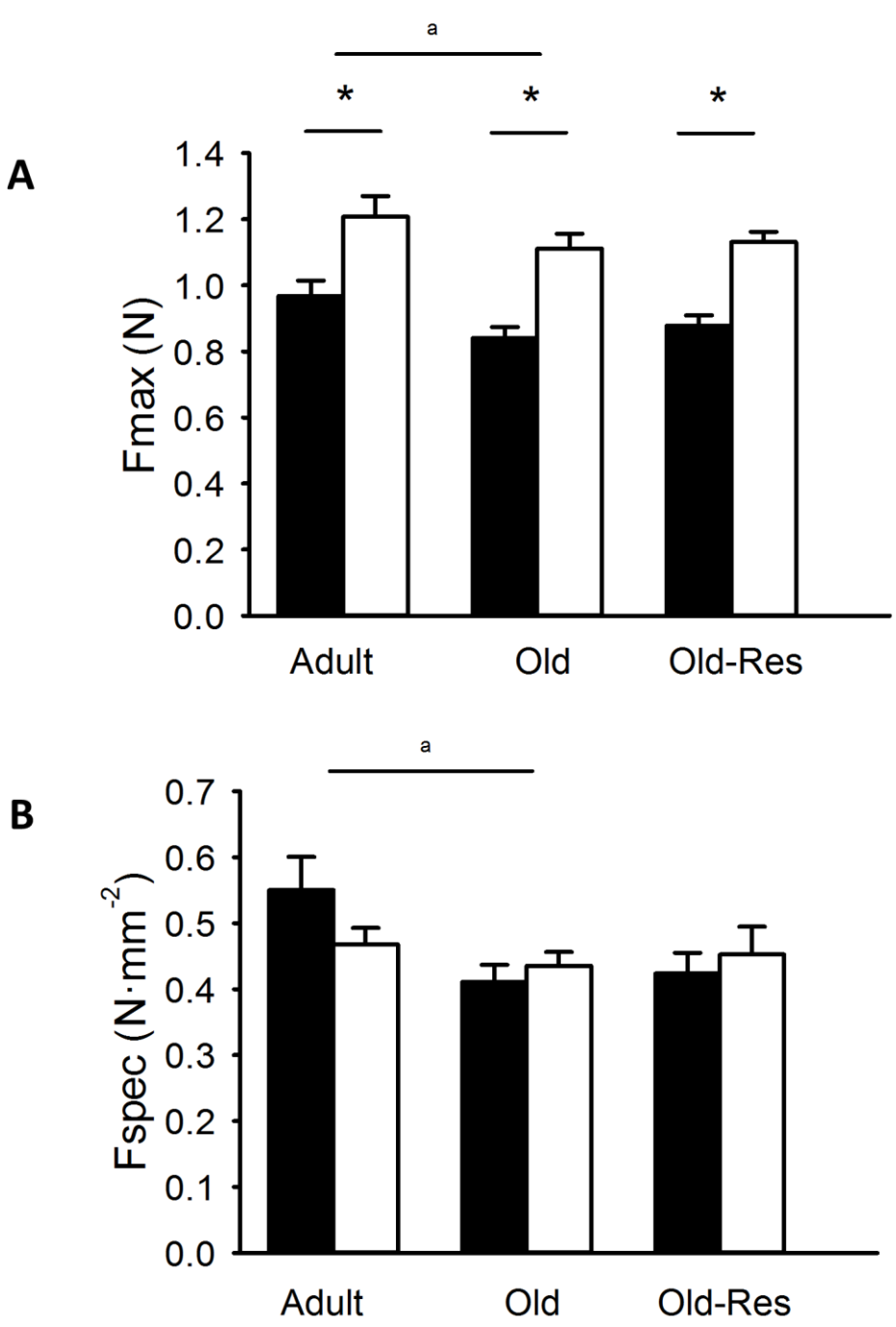
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Fig. 1

2

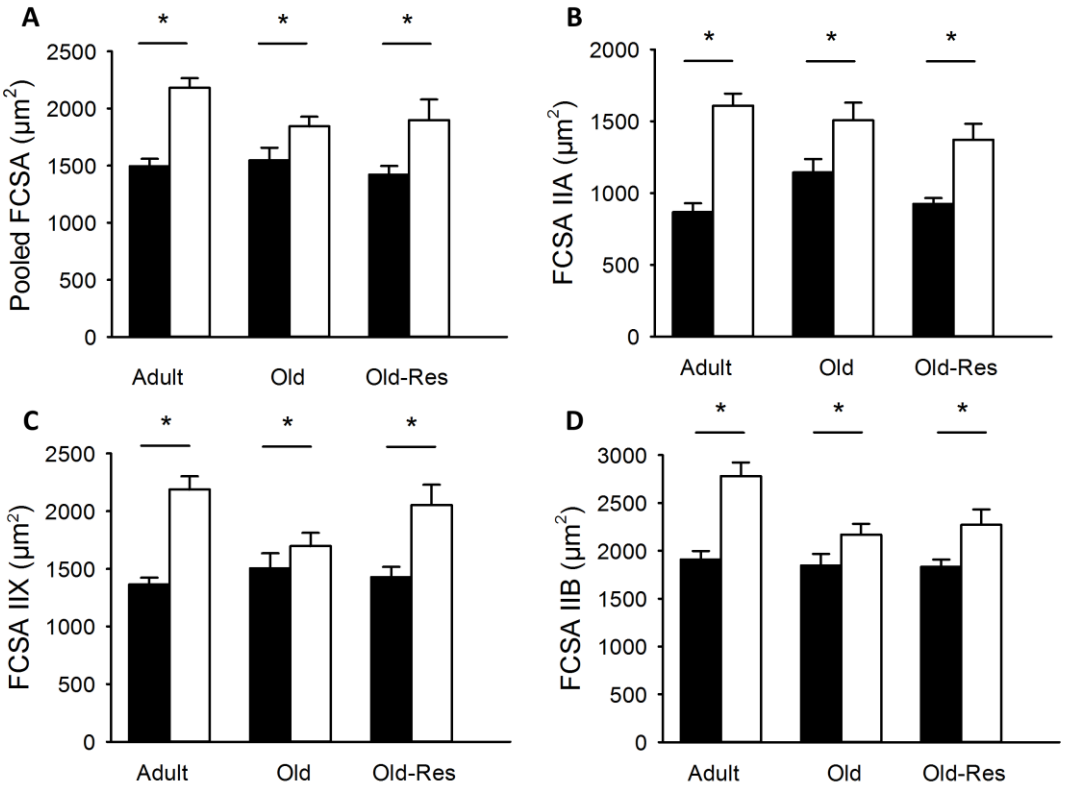

Fig. 2

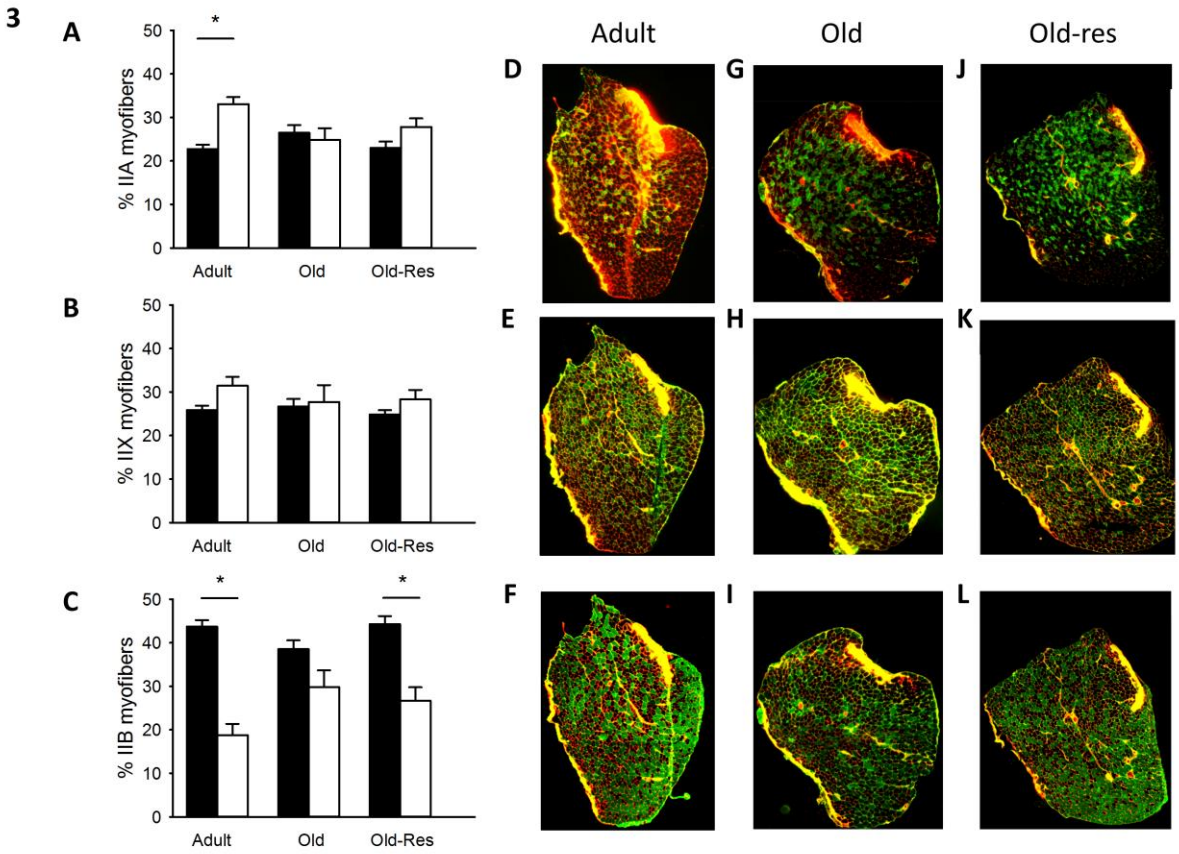

Fig. 3

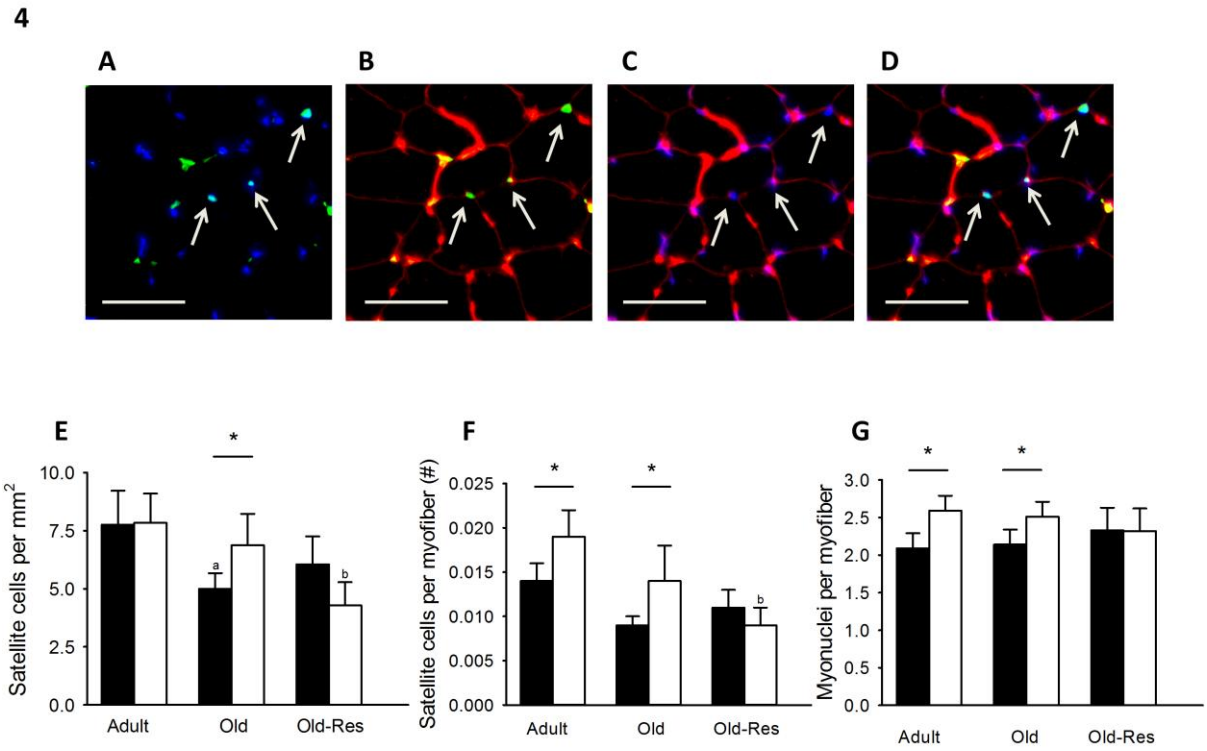

Fig. 4

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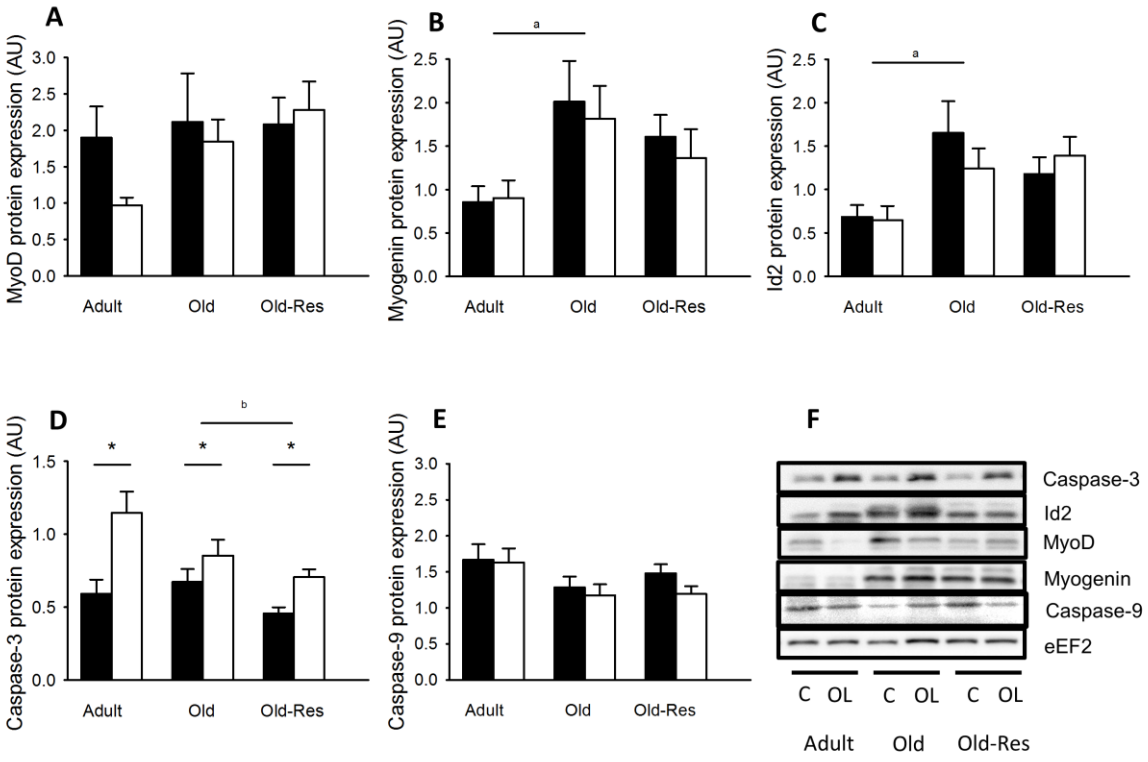

Fig. 5

Highlights

- The hypertrophic response was blunted in 25-month-old compared to 9-month-old mice
- The blunted hypertrophic response was characterized by blunted myofiber hypertrophy
- The lower number of SCs per CSA may underlie the blunted myofiber hypertrophy
- Resveratrol did not alleviate the age-related blunted hypertrophic response
- Resveratrol abolished the increase in SC content after overload